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14. ABSTRACT

This grant was awarded to the PI at Louisiana State University. He left there in Aug 2006 and with the assistance of Grant Manager, Dr. Carole Christian, it was transferred to the University of Louisville where he started Apr 2007. Minimal work was done on the project prior to departure from LSU and minimal funds were expended.

This project was essential to my obtaining an appointment as an Associate Professor of Medicine at the University of Louisville's James Graham Brown Cancer Center, along with significant startup package, and my involvement as a Project PI on the Brown Cancer Center's NIH-funded Molecular Targets Program.

Currently, my new laboratory has been equipped with the needed computer hardware and software. I anticipate the postdoctoral fellow hired to participate on the project will start by Aug 6, 2007, and given the revised timeline for this project, I expect that it will be completed on time and as planned.

15. SUBJECT TERMS
Structure-activity relationship modeling, proteomics, drug discovery

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Introduction

The objective of this project is to employ an innovative approach to discover new molecular targets found only in estrogen receptor positive (ER+) breast cancer cells that are 1) highly associated with cell type-specific toxicity, 2) compounds that influence or interact with them, and 3) ultimately anticancer pharmacophores that uniquely target breast tumor cells to be used as the basis for the design of new anticancer drugs. We observed that certain chemicals display potent toxicity to one type of breast cancer cell line and not other related lines. Based on this observation, the **hypothesis** for the project is that this cell type-specific toxicity is due to an interaction of a chemical agent with a specific molecular target found only in the sensitive cells. The project is defined by two working hypotheses. The **first working hypothesis** is that congeneric sets of compounds that display this excessive and specific toxicity to ER+ cells will influence particular molecular targets found only with the sensitive cell line. The reasoning for this is based on the accepted premise of SAR modeling that like structure begets like activity. The **second working hypothesis** is that, when the proteome of the ER+ cell line is probed with a defined congeneric series of compounds that display this cell type-specific activity, these compounds will all affect, minimally, the same identifiable molecular target. Through the techniques of comparative proteomics, we anticipate being able to identify these unique target(s) and thus provide the basis for highly effective antibreast cancer therapies.

Body

As mentioned in the previous Annual Report, this grant was awarded to the PI at Louisiana State University. He left there in Aug 2006 and with the assistance of Grant Managers Drs. Christian and Fallas, the grant was transferred to the University of Louisville where the PI started Apr 2007. Minimal work was done on the project prior to departure from LSU and minimal funds were expended. This project was essential to the PI obtaining an appointment as an Associate Professor of Medicine with a joint appointment as Associate Professor of Pharmacology and Toxicology at the University of Louisville's James Graham Brown Cancer Center, along with significant startup package, and involvement as a Project PI on the Brown Cancer Center's NIH-funded Molecular Targets Program.

Currently, the PI's new laboratory has been equipped with the needed computer hardware and software described in the proposal. In August 2007, Shahid Qamar was hired to fill the postdoctoral fellow position. At the time, Shahid was nearing the completion of his PhD studies at Arizona State University and he plans to now defend his dissertation in October 2008.

The specific aims for the project are:

1. Model Development: Create high-quality "control" SAR models for breast cancer cell lines based on 50% growth inhibition, total growth inhibition, and 50% lethal concentration (GI_{50}, TGI, and LC_{50}) values and "experimental" excessive toxicity models based on compounds that display potent and specific toxicity to ER+ cells and minimal toxicity to other comparable cell types.

2. Pharmacophore Identification: Based on structural information developed in Aim 1, identify congeneric sets of compounds associated with excessive and specific toxicity to ER+ cell lines. Define and fine-tune pharmacophores from these models with ligand-based three dimensional SAR methods. Based on these pharmacophores, develop small libraries of available and suitable compounds for in vivo testing. Verify the association between the pharmacophore and defined cell toxicity in vivo with selected compounds.

3. Proteome Target Identification: Using protein mass spectrometric techniques (i.e., proteomics), identify the specific molecular targets associated with excessive and specific toxicity to ER+ cells that are influenced by these pharmacophore-defined sets of compounds and ascertain their involvement in ER+ -cell type-specific toxicity.

Specific Aim 1:

As described in the proposal, the project is based on the observation that certain chemicals display potent toxicity to one type of breast cancer cell line and minimal or no toxicity to other related ones. By comparing chemicals that exhibit this "excess" or "differential" toxicity to one line and not another, the project's goal is to identify what is interesting or critical about these chemicals that gives rise to cell type-specific toxicity. Our criteria for chemical identification and subsequent modeling and target identification is greater toxicity toward estrogen receptor (ER) positive (ER+) cell lines than other similar cell lines. The project will start with comparisons between ER+ MCF-7 breast cancer tumor cells and ER negative (ER-) MDA-MB-231 ones and vice versa. This may seem to overly simplify the problem and suggest that we may rediscover the ER. However, absence or presence of the ER is only one attribute separating MCF-7 from MDA-MB-231 cells. We know that the ER and its signaling pathway are associated with many components including regulators, cofactors, metabolic enzymes, and transport mechanisms. Thus the method allows for the identification of agent-target interactions at numerous points associated with ER status.

All of the proposed models have been developed with the cat-SAR structure-activity relationship (SAR) program as described in the proposal. These include MCF-7, MDA-MB-213 models for GI50, TGI, and LC50 and the projects' key models, the excessive toxicity models (i.e., MCF-7 – MDA-MB-213 and MDA-MB-231 – MCF- 7). A total of nine models were developed with each model consisting of 400 compounds selected from the DTP with the desired activity (i.e., potency for the MCF-7 and MDA-MB-231 models and excessive toxicity to only one cell line for the MCF-7 – MDA-MB-231 and MDA-MB-231 – MCF-7 models).

First, a series of range-finding experiments were carried out in order to select the best overall modeling parameters. These included HQSAR 2-D fragment lengths and cat-SAR modeling parameters (e.g., number of chemicals and proportions of active and inactive compounds required to select important fragments). These included models for HQSAR fragment length of three to seven, and ones with single fragment lengths from seven to 12 heavy atoms. We selected the eight heavy atom fragment size models for further work since 1) models built on them were able to predict a significant number of compounds from the learning sets and eight heavy atoms is an appropriate size for developing pharmacophores for later 3-D QSAR modeling. Moreover, from a practical point, models of atom size 12 had roughly nearly 200000 fragments wherein size eight had about 84000. This reduction in fragments made data management and analysis more practical without losing predictive ability.

During the preliminary validation exercise, a self-fit of each model was accomplished wherein each developed model was used to predict the activity of compounds in the model. The concordance between experimental and predicted activity ranged between 83% and 97% (Table 1).

Table 1. Self-fit model validation summary.

Model		Sensitivity	Specificity	Concordance
GI50	**MDA**	0.905(181\200)	0.888(175\197)	0.897(356\397)
	MCF	0.915(183\200)	0.894(177\198)	0.905(360\398)
	MDA-MCF	0.951(117\123)	0.741(63\85)	0.865(180\208)
	MCF-MDA	0.862(150\174)	0.787(133\169)	0.825(283\343)
LC50	**MDA**	0.913(179\196)	0.943(182\193)	0.928(361\389)
	MCF	0.944(170\180)	0.752(109\145)	0.858(279\325)
	MDA-MCF	0.964(163\169)	0.877(136\155)	0.923(299\324)
	MCF-MDA	0.984(179\182)	0.949(168\177)	0.967(347\359)
TGI	**MDA**	0.894(178\199)	0.943(183\194)	0.919(361\393)
	MCF	0.874(174\199)	0.898(177\197)	0.886(351\396)
	MDA-MCF	0.887(134\151)	0.884(107\121)	0.886(241\272)
	MCF-MDA	0.952(177\186)	0.966(173\179)	0.959(350\365)

Footnotes:
Sensitivity: number of correct positive predictions / total number of positive predictions.
Specificity: number of correct negative predictions / total number of negative predictions
Concordance: Observed Correct Predictions: number of correct predictions / total number of predictions
Values in parentheses: (number correct prediction / number of predictions)

Table 2. Leave-one-out model validation summary.

Model		Sensitivity	Specifity	Concordance
GI50	**MDA**	0.81(162\200)	0.909(179\197)	0.859(341/397)
	MCF	0.755(151\200)	0.904(179\198)	0.829(330/398)
	MDA-MCF	0.697(83\119)	0.68(70\103)	0.689(153/222)
	MCF-MDA	0.657(109\166)	0.854(152\178)	0.759(261/344)
LC50	**MDA**	0.746(147\197)	0.891(172\193)	0.818(319/390)
	MCF	0.815(145\178)	0.705(105\149)	0.765(250/327)
	MDA-MCF	0.881(148\168)	0.671(110\164)	0.777(258/332)
	MCF-MDA	0.652(92\141)	0.875(140\160)	0.771(232/301)
TGI	**MDA**	0.879(175\199)	0.802(154\192)	0.841(329/391)
	MCF	0.793(157\198)	0.878(173\197)	0.835(330/395)
	MDA-MCF	0.803(118\147)	0.743(101\136)	0.774(219/283)
	MCF-MDA	0.737(132\179)	0.769(143\186)	0.753(275/365)

Footnotes: see Table 1

The leave-one-out (LOO) validations consisted of each chemical, one at a time being removed from the model's learning set. The model was rederived with the n-1 set, and then used to predict the activity of the chemical left out of the results. The concordance for these models ranged between 75 and 86% (Table 2). These values from our cat-SAR program are slightly better than those expected based on some of the preliminary findings described in the proposal where in concordance of GI50 models built with the MCASE system ranged between 72 and 84%.

With respect to the success of Specific Aim 1, a manuscript is being prepared for publication.

Specific Aim 2:

To begin, we selected the compounds in the MCF-7 – MDA-MB-231and MDA-MB-231- MCF-7 GI50 models with the highest difference in potency between the two cell lines that were correctly predicted in the LOO validations. Essentially, this assured an unbiased (and correct) association of fragments (pharmacophores) to chemicals for QSAR analyses.

For the MCF-7 - MDA-MB-231 models we selected compounds NSC 625587, 663791, and 139105 with differences in potencies between MCF-7 and MDA-MB-231 of 3.30, 3.29, and 3.25, respectfully. We note that compounds with the highest difference in potencies were compounds NSC 674496 and 674495 (3.78 and 3.46, difference in potencies respectfully) were not predicted in the cat-SAR LOO and hence not selected for QSAR studies. Next, cat-SAR model fragments were selected that were derived from about 10 other molecules in the database. These fragments and compounds then formed the basis for a preliminary CoMFA (Comparative Molecular Field Analysis) and CoMSIA (Comparative Molecule Similarity Index—a similar modeling approach to CoMFA).

The preliminary CoMFA or CoMSIA models for NSC 625587 and 139105 after employing the routine of focusing the model netted an r2 = 1.00 and 0.99, respectfully and a q2 = 0.63 and 0.51, respectfully. See Table 3 and Figure one for initial depiction of QSAR model for compound NSC 625587.The q2 value produced from a CoMFA analysis is essentially a cross-validated r2. We note that Tripos mentions that q2 $\geq$.0.5 are likely to be useful in decision making processes. As such, NSC 663791 did not produce a meaningful model.

Table 3. Pharmacophore/compound selection for Comparative Molecular Field Analysis for compound 625587.

NSC	MCF-7 GI_{50}	MDA-MB-231 GI_{50}	Difference	SAR Classification
256439	8.33	6.59	1.74	Positive
267469	8.27	6.63	1.63	Positive
284682	6.81	5.06	1.75	Positive
625587	7.74	4.44	3.3	Positive
628676	7.34	4.90	2.43	Positive
644945	7.31	5.58	1.73	Positive
650931	8.41	6.63	1.78	Positive
683416	7.32	4.41	2.91	Positive
683557	7.94	5.15	2.78	Positive
707850	4.74	4.75	0.003	Negative

For the MDA-MB-231 – MCF-7 models we selected compounds NSC 695065, 713079, and 342913 with differences in potencies between MDA-MB-231 and MCF-7 of 2.27, 2.69 1.90. We note that the compounds with highest differences in potencies were NSC 148823, 72055, and 695065 (3.40, 2.86, 2.69, difference in potencies, respectfully) were not predicted in the cat-SAR LOO and hence not selected for QSAR studies. The preliminary CoMFA or CoMSIA models for NSC 695065 and 342913 after employing the routine of focusing the model netted an r2 = 1.00 and 0.99 respectfully and a q2 = 0.63 and 0.51 respectfully. NSC 713079 did not produce a meaningful model.

It should be mentioned that the failure of some chemicals to model properly was expected and was essentially one of the key points to Specific Aim 2, wherein the aim was designed to weed-out potentially troublesome chemicals for subsequent proteomic analyses. Hence, regarding Specific Aim 2, we feel confident that we will be able to use CoMFA and CoMSIA modeling as described in the proposal to help identify the best suited candidates for further analyses. With the apparent success of Specific Aim 2 (i.e., the use of cat-SAR fragments as pharmacophores for CoMFA and CoMSIA modeling as well as being able to obtain QSAR for chemicals with highly different potencies with regards to two related cell lines) we are in the initial stages of organizing a manuscript for publication. We also note that we are slightly behind schedule from our Statement of Work that indicated we would be passing recommended compounds for in vitro testing to our collaborator Dr. Day for proteomic analyses. We estimate that we will be providing him with data for proteomic analyses with the next month or so.

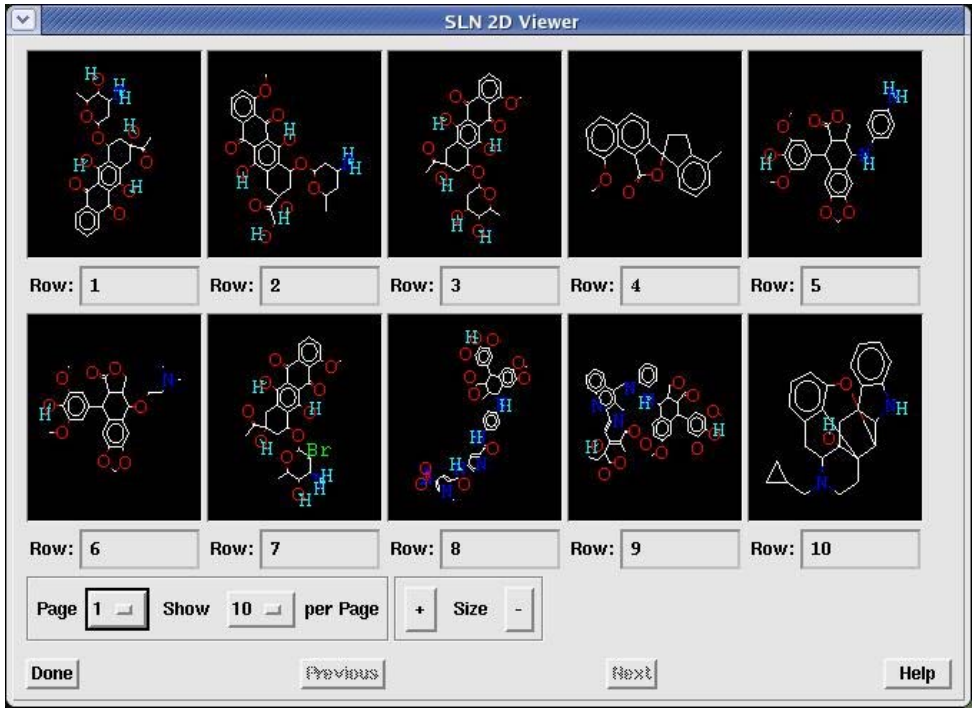

Figure 1. Chemical structures used for QSAR analysis for compounds NSC625587

For Specific Aim 3: Proteome Target Identification, this specific aim as described in the statement of work will entail differential proteomics analysis between breast cancer cell lines that respond differentially to cytostatic and cytotoxic agents. Over the past year, while Specific Aims 1 and 2 were being worked on by the PI, Dr. Day (collaborator at the University of Pittsburgh) has been determining differentially expressed proteins in MCF-7 and MCF-7/LY2 cells exposed to tamoxifen. Essentially, Dr. Day has been readying the experimental processes required for Specific Aim 3.

With regard to Specific Aim 3, following is an abstract presented at the 2008 American Association for Cancer Research (see also Appendix AACR poster):

Approximately two million women currently living in the United States have been treated for breast cancer. Last year, an estimated 212,920 women were diagnosed with, while an additional 40,970 women were expected to die from the disease. About 70% of breast cancer cases diagnosed are estrogen receptor positive (ER+). Tamoxifen, a selective estrogen receptor modulator in use since the 1970's, is the most common treatment for women with ER+ breast cancer or those who are at risk for breast cancer. One of the major problems associated with tamoxifen is acquired resistance to the drug. The majority of tumors initially responding to tamoxifen eventually develop resistance to endocrine therapy and progress. There are several proposed mechanisms to account for this resistance, yet the phenomenon is still not fully understood. In this study, we used several proteomic methods to better understand tamoxifen resistance. The MCF-7 breast carcinoma cell line and its antiestrogen-resistant derivative, MCF-7/LY2, were used to begin determination of the proteins differentially expressed between the two, and the role such proteins may play in tamoxifen resistance. Initially, analysis was performed on whole cell lysates. The first relative quantitation method used was the "isobaric tags for relative and absolute protein quantitation (iTRAQ)" approach. Four different cell lysates, MCF-7 and MCF-7/LY2, untreated and tamoxifen-treated, were prepared and tagged. Labels were alternated between replicates to account for variation. Proteins were then identified and quantified using LC/MS-MS with a MALDI-TOF/TOF-MS and an ESI-qQTOF-MS system. The second method used was difference 2D gel electrophoresis (DIGE), which employs two electrophilic Cy dyes to label the samples to be compared in the same gel. Several heat shock proteins (HSPs) were found at differing levels in the two lines, including HSP10, HSP27, HSP60, and HSP70. HSP27 found to be present at ca. 2-fold higher levels in the antiestrogen-resistant MCF-7/LY2 cell line. The level of this protein, originally identified in MCF-7 cells, is influenced by estrogens. HSP27 has also been shown to be increased in breast cancer and to correlate with the ER+ status. The synthesis of HSP27 has also been shown to occur with the development of drug resistance, including that of breast cancer cells to doxorubicin. Another interesting protein, found to be decreased ca. 2-fold in the MCF-7/LY2 cells, was macrophage migration inhibitory factor (MIF1). MIF1 has an interesting relationship to antiestrogen resistance because of its role in regulating transcription from an AP-1 site. Other proteins found to be present in lower levels in the antiestrogen-resistant line included triosephosphate isomerase and alpha-enolase. Identification of proteins differentially

regulated in tamoxifen responsive and resistant lines will help to understand why cells develop resistance to antiestrogens. Follow-up work on the proteins identified in this study may lead to the development of potential biomarkers or new therapeutic strategies.

Hence, specific to the goals of this project, it is clear from the work of Dr. Day that once interesting compounds from SA2 are passed along for proteomic analysis, the mechanisms and techniques are in place to succinctly process the compounds and analyze the results for potentially new therapeutic targets for breast cancer drug discovery.

Key Research Accomplishments

As described above

- SAR models have been produced and validated for MCF-7 and MDA-MB-231 GI_{50}, TGI, and LC_{50} individually and for differential activity when comparing MCF-7 and MDA-MB-231 cells. Hence, SA1 is completed.
- The first CoMFA/CoMSIA models for SA2 have been preliminarily analyzed. Results suggest that the proposed use of CoMFA/CoMSIA 3-dimensional modeling will be useful in the decision making process for selecting the most promising compounds for proteomic analyses in SA3.
- The tools and techniques to accomplish SA3 have been worked over in the Day lab with all indications that proteins differentially expressed in breast cancer cells line exposed to the same chemicals will be realized.

Reportable Outcomes

1. Poster: Miranda J. Sarachine, Tamanna Sultantana, Mirunalni Thangavelu, Manimalha Balasubramani, and Billy W. Day, Identification and Quantitation of Differentially Expressed Proteins Proteins in Tamoxifen-Resistant Breast Cancer Cells, Presented at the American Association for Cancer Research 2008 Annual Meeting.
2. Manuscript in preparation: Shahid Qamar and Albert R. Cunningham. Structure-Activity Relationship Model for Differential Growth Inhibition of MCF-7 and MDA-MB-231 Cells.
3. Manuscript in preparation: Miranda J. Sarachine, Tamanna Sultantana, Mirunalni Thangavelu, Manimalha Balasubramani, Albert R. Cunningham, and Billy W. Day. Identification and Quantitation of Differentially Expressed Proteins Proteins in Tamoxifen-Resistant Breast Cancer Cells.
4. The list of SAR databases outlined in Tables 1 and 2 have been produced and validated.
5. As noted in the last Annual Report, the PI was able to successfully use the results from his prior Idea award and the applicability of this Idea award to obtain appointments as an Associate Professor of Medicine and Associate Professor of Pharmacology and Toxicology at the University of Louisville's School of Medicine as well as an appointment to its James Graham Brown Cancer Center. Regarding the Brown Cancer Center, in September 2003 it was awarded a five-year, $11 million Center of Biomedical Research Excellence (COBRE) grant from the National Center for Research Resources at the National Institutes of Health under the directorship of Dr. Donald Miller. The grant established the Molecular Targets Program, which the PI is part of by providing for the recruitment of researchers from a variety of disciplines to identify and develop new molecular targets for anti-cancer drugs and therapies using the techniques of modern structural biology. Since I was originally recruited into this program at the end of its five year period, Dr. Miller submitted the project for renewal wherein this BCRP award was mentioned in the renewal application and the COBRE grant to Dr. Miller has been renewed
6. Miranda J. Sarachine, as mentioned above is a current BCRP Predoctoral Fellow in the Day lab at the University of Pittsburgh. Ms. Sarachine is a School of Medicine graduate student who will working on this project and is being partially funded through this grant's subcontract with the Unversity of Pittsburgh.

Conclusion

To date, with the series of well-working models developed in SA1 that are capable of analyzing not only cytostatic and cytotoxic activity to MCF-7 and MDA-MB-231 cells but also describing differences in activities induced by small molecules to these cell lines, we are confident that this project is on the right course to achieve the goal set out in the proposal, i.e., the identification of molecular targets and pharmacophores that are exquisitely associated with high toxicity and high cell type-specificity. Furthermore, with the success of the preliminary CoMFA and CoMSIA modeling, we are confident that we will be able to fine-tune the selection process of compounds for proteomics analysis. In essence, the initial success of SA2 indicates that this 3-dimenstional QSAR modeling approach will allow to remove, prior to in vitro testing, potential outliers of the process. And finally, with the initial work conducted by Dr. Day with regards to protein identification in different breast cancer cell lines exposed to the same compounds, we are confident that SA3 will not encounter any significant pitfalls.

References

None

Appendices:
AACR Poster

Identification and Quantitation of Differentially Expressed Proteins Proteins in Tamoxifen-Resistant Breast Cancer Cells

Miranda J. Sarachine,[1] Tamanna Sultantana,[2] Mirunalni Thangavelu,[2] Manimalha Balasubramani,[2] and Billy W. Day[3]

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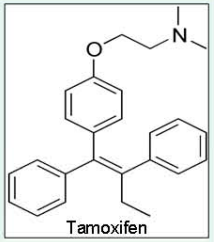
ABSTRACT

Approximately two million women currently living in the United States have been treated for breast cancer. Last year, an estimated 212,920 women were diagnosed with, while an additional 40,970 women were expected to die from the disease. About 70% of breast cancer cases diagnosed are estrogen receptor positive (ER+). Tamoxifen, a selective estrogen receptor modulator in use since the 1970's, is the most common treatment for women with ER+ breast cancer or those who are at risk for breast cancer. One of the major problems associated with tamoxifen is acquired resistance to the drug. The majority of tumors initially responding to tamoxifen eventually develop resistance to endocrine therapy and progress. There are several proposed mechanisms to account for this resistance, yet the phenomenon is still not fully understood. In this study, we used several proteomic methods to better understand tamoxifen resistance. The MCF-7 breast carcinoma cell line and its antiestrogen-resistant derivative, MCF-7/LY2, were used to begin determination of the proteins differentially expressed between the two, and the role such proteins may play in tamoxifen resistance. Initially, analysis was performed on whole cell lysates. The first relative quantitation method used was the "isobaric tags for relative and absolute protein quantitation (iTRAQ)" approach. Four different cell lysates, MCF-7 and MCF-7/LY2, untreated and tamoxifen-treated, were prepared and tagged. Labels were alternated between replicates to account for variation. Proteins were then identified and quantified using LC/MS-MS with a MALDI-TOF/TOF-MS and an ESI-qQTOF-MS system. The second method used was difference 2D gel electrophoresis (DIGE), which employs two electrophilic Cy dyes to label the samples to be compared in the same gel. Several heat shock proteins (HSPs) were found at differing levels in the two lines, including HSP10, HSP27, HSP60, and HSP70. HSP27 found to be present at ca. 2-fold higher levels in the antiestrogen-resistant MCF-7/LY2 cell line. The level of this protein, originally identified in MCF-7 cells, is influenced by estrogens. HSP27 has also been shown to be increased in breast cancer and to correlate with the ER+ status. The synthesis of HSP27 has also been shown to occur with the development of drug resistance, including that of breast cancer cells to doxorubicin. Another interesting protein, found to be decreased ca. 2-fold in the MCF-7/LY2 cells, was macrophage migration inhibitory factor (MIF1). MIF1 has an interesting relationship to antiestrogen resistance because of its role in regulating transcription from an AP-1 site. Other proteins found to be present in lower levels in the antiestrogen-resistant line included triosephosphate isomerase and alpha-enolase. Identification of proteins differentially regulated in tamoxifen responsive and resistant lines will help to understand why cells develop resistance to antiestrogens. Follow-up work on the proteins identified in this study may lead to the development of potential biomarkers or new therapeutic strategies.

BACKGROUND

- According to the American Cancer Society, in 2007[1]:
  - 18,480 women will be diagnosed with invasive breast cancer and 62,030 with in situ disease
  - 40,460 women will die from breast cancer
- About 70% of diagnosed breast cancer cases are ER+[2]
- Tamoxifen, a selective estrogen receptor modulator, is the drug of choice for ER+ breast cancer cases
  - ER antagonist in the breast, which blocks estrogen stimulation of breast cancer cells
  - De novo and acquired resistance are a problem
    - Almost 50% of patients with advanced disease do not respond
    - 40% of patients treated experience tumor relapse and die
    - Several proposed mechanisms, but not fully understood [2]



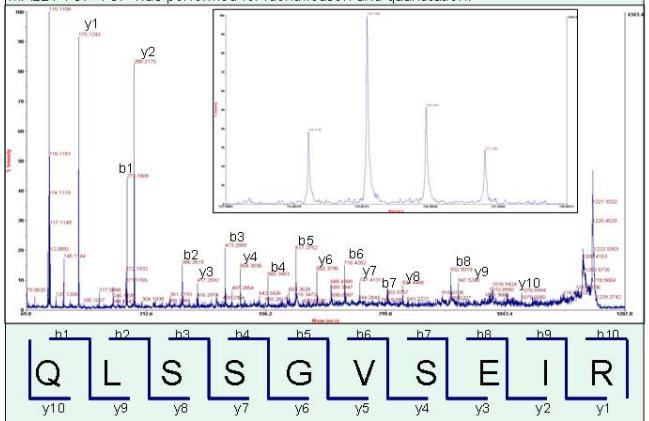
- One study has examined sensitive and resistant xeno-transplanted tumors through 2-DE
  - Proteins involved in signal transduction, mitochondrial respiratory chain and oxidative stress processes[3]
- Goal: Use proteomics-based methods (DIGE and iTRAQ) to identify differentially expressed proteins between tamoxifen resistant and responsive breast cancer cell lines

METHODS

- MCF-7 and MCF-7/LY2 cells + 10 μM Tamoxifen or DMSO as the vehicle control for 48 hours
- Whole cell lysates of these cells were prepared, and iTRAQ and DIGE were performed

iTRAQ™

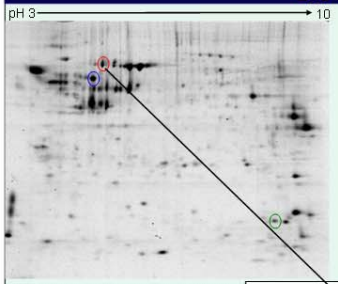
Whole cell lysates (100 μg of protein) were reduced, alkylated, and digested with trypsin. The peptides were then labeled with the iTRAQ™ reagents. These are amine-specific isotobaric tagging reagents that label peptides at the N-terminus and lysines. The labeled peptides look the same in MS, but when fragmented, the label disassociates and the reporter can be seen. One trial consisted of: MCF-7 (117), MCF-7 + Tam (116), MCF-7/LY2 (115), MCF-7/LY2 + Tam (114). The second trial reversed the labels with: MCF-7 (114), MCF-7 + Tam (115), MCF-7/LY2 (116), MCF-7/LY2 + Tam (117). All samples in each trial were then combined and fractionated by strong cation exchange chromatography using PolySulfoethyl A Macro spin columns. The fractions were then separated further on a reverse phase C18 column, collected with a ProBot onto stainless steel targets, and MALDI-TOF-TOF was performed for identification and quantitation.



Above is an example MS-MS spectrum of one of the peptides of HSP27. Below the spectrum is the amino acid sequence demonstrating the b and y ions. Below is a table containing some of the proteins identified as differentially expressed in the iTRAQ experiments. The proteins in this table were identified by two or more peptides. The quantitation is the averaged ratio for all peptides identified for the protein.

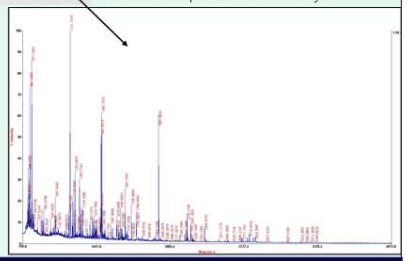
Protein	MCF-7 +Tam : MCF-7	MCF-7/LY2 : MCF-7	MCF-7/LY2 + Tam : MCF-7
Alpha-enolase	2.2	2.9	0.81
Cyclophilin A	1.8	4.9	1.0
HSP 27	1.6	2.4	1.2
HSP 70	3.4	4.1	0.58
MIF-1	1.2	1.3	0.47
Nucleolin	1.7	2.2	0.56
Protein disulfide-isomerase A3	0.62	2.3	2.3
Protein DJ-1	2.1	1.6	0.50
Pyruvate kinase isozymes M1/M2	2.2	2.9	0.55
Triosephosphate isomerase	3.0	3.5	0.65

DIGE



Whole cell lysates of MCF-7 or MCF-7/LY2 treated with tamoxifen or DMSO were separately labeled with Cy3 or Cy5, combined as comparative pairs, and separated first by isoelectric focusing on pH 3-10 (NL) IPG strips. Then the strips were loaded onto 8-16% Tris-HCl gels and separated by molecular radius. Reciprocal labeling was done with each pair of samples. This gel is the Cy5 image of the whole cell lysate of MCF-7/LY2 cells, with the DMSO-treated sample labeled with Cy5 and tamoxifen-treated sample labeled with Cy3.

Differentially expressed proteins were identified using DeCyder™ software. The spots were excised, digested with trypsin, and spotted onto MALDI target plates. MALDI-TOF-TOF-MS was then performed. The proteins that correspond to those identified in iTRAQ are HSP70 (red), HSP60 (blue), cyclophilin A (green). The peptide mass fingerprint of HSP70 is seen at the right.



CONCLUSIONS

- Most of the proteins identified correspond to those previously identified as differentially expressed in another study wherein the MaCa 3366 tamoxifen-sensitive xenograft and the MaCa 3366/TAM tamoxifen-resistant xenograft were compared by 2-DE[3]
- One of the more interesting proteins is HSP27
  - First described as an estrogen-regulated protein and is significantly associated with with the ER in breast cancer[4]
  - Previously identified to be overexpressed in breast cancer cells resistant to doxorubicin[5]
- MIF-1
  - Cytokine produced by macrophages and involved in the anti-inflammatory response
  - Role in growth regulation and cell cycle control - interacts with Jab-1, a co-activator af AP-1 regulated transcription[6]
  - Demonstrated in the past to be overexpressed frequently in primary breast cancers and sera[7]
- Further investigation into the proteins initially identified in this study may help to identify biomarkers of tamoxifen resistance and new therapeutic targets

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